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Growth factor-induced release of a glycosyl-phosphatidylinositol (GPI)-linked protein from the HEp-2 human carcinoma cell line

Julia M. Roberts, Paul Kenton and Peter M. Johnson

Department of Immunology, University of Liverpool, Liverpool, UK

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The release of a GPI-linked oncofetal protein (PLAP) from HEp-2 cells has been studied as an indicator of endogenous PI-specific PLC activation. A non-hydrolysable GTP analogue, EGF and insulin all produced a dose-dependent release of PLAP from these cells. This indicates that an increase in PLC activity following either growth factor binding or G protein activation may cause cellular release of PLAP.

GPI-linked protein; Placental type alkaline phosphatase; Growth factor; Tumor cell

1. INTRODUCTION

Human placental syncytiotrophoblast after the first trimester of pregnancy expresses placental-type alkaline phosphatase (PLAP) on its cell surface together with several growth factor receptors [1,2]. PLAP is a dimeric 130 kDa sialoglycoprotein that is also expressed by many tumour cell lines (including HEp-2) and solid tumour tissues [1,3–5]. PLAP has been shown to be one of an increasing number of cell surface molecules anchored to the outer leaflet of the plasma membrane by a glycosyl-phosphatidylinositol (GPI)-linkage that can be specifically cleaved by bacterial phosphatidylinositol-specific phospholipase-C (b-PLC) [4,7].

Many tumour cell lines are known to express high numbers of growth factor receptors (e.g. epidermal growth factor (EGF) receptor on HEp-2 cells (Roberts, J.M., unpublished observations)). The EGF, insulin and platelet-derived growth factor (PDGF) receptor are among the best characterised and all possess ligand-induced intrinsic tyrosine-specific kinase activity, which is an essential part of their signal transduction mechanisms [6]. An important early mediator of increased cellular phosphatidylinositol (PI) turnover following the interaction of cell surface growth factor receptors with their corresponding ligand is the activation of endogenous cellular PI-hydrolysing enzymes, collectively termed PLC [8]. PLC may be coupled to certain cell surface receptors by a group of G proteins [9], such has been demonstrated for vasopressin, bombesin, bradykinin, α thrombin, thyrotropin-releasing hormone and angiotensin II [9,10]. However, recent evidence suggests that an alternative mechanism for PLC activation is through the PLC phosphorylation by growth factor receptor-tyrosyl kinase (RTK) complexes [11–13]. We have studied PLAP release as an indicator of endogenous PLC activation through both RTK and G protein activation in the HEp-2 human carcinoma cell line.

Correspondence address: J.M. Roberts, Department of Immunology, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

2. MATERIALS AND METHODS

2.1. *Cell culture*

The HEp-2 (human laryngeal carcinoma) cell line was maintained in minimal essential medium containing 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). HEp-2 cells were grown to confluence in 6-well plates (approximately 1×10^6 cells per well) and then incubated in serum-free medium for 2 h before use. Cells were then washed 3× in Hanks' balanced salt solution (HBBS), pH 7.4, containing 20 mM Hepes. A well of cells was trypsinised and counted on the day of each experiment to give a cell count.

2.2 *Treatment with b-PLC*

Hep-2 cells were incubated with 0.025 U/ml (lowest optimum concentration) of purified PI-specific PLC (PI-PLC) from *Bacillus thuringiensis* (Peninsular Laboratories, Merseyside) in isotonic phosphate-buffered saline (PBS), pH 7.4, for 1 hour at 37°C, or with a PLC specific for phosphatidylcholine from *Clostridium perfringens* (Sigma type XII), or buffer alone. The incubation medium was microfuged at 11 000×g for 10 min and the supernatant stored at −20°C or assayed for PLAP. The PLAP (mU/ml) released into the buffer alone was subtracted as background from all other experimental values when calculating results.

2.3. *Incubation with GTP analogues*

GTPγS tetralithium salt and GDPβS trilithium salt (Sigma) were stored at −20°C. HEp-2 cells were treated with 25 μg/ml freshly dissolved digitonin in HBBS/Hepes for 5 min at room temperature to permealyse the cells. This was followed by 3 washes with HBBS/Hepes and then the cells were incubated with HBBS/Hepes alone, GTPγS or GDPβS for 30 min at 37°C. The incubation medium was collected as for PLC treatment.

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2.4. *Incubation with growth factors*

Following incubation with serum-free medium, cells were washed and incubated with insulin (range 25 μg/ml to 10 ng/ml, porcine, Sigma), EGF (250- 1 ng/ml, human recombinant, Boerhinger Mannheim) or PDGF (100- 1 ng/ml, porcine Boerhinger Mannheim) in 1 ml HBBS/Hepes at 37°C for 2 h. Cells incubated in buffer alone were used as controls. The incubation medium was collected and assayed for PLAP as before.

2.5. *PLAP assay*

PLAP release was measured by a sensitive enzyme immunoassay using the H317 murine monoclonal antibody (mAb) specific for human PLAP or the H315 mAb specific for both PLAP and PLAP-like enzymes [1,14,15]. Assays were standardised using known amounts of purified human PLAP (Sigma). Undiluted cell-free super natants (150 μl), with Tween 20 added to 0.05% were assayed fo PLAP in triplicate.

3. RESULTS

3.1. *PLC-induced PLAP release from HEp-2 cells*

Treatment of HEp-2 cells with PI-specific PLC released significant amounts of PLAP into the cell culture supernatant compared with controls (Table I). When the released material was concentrated 6-fold and applied to SDS-PAGE gels under non-reducing conditions and Western blotted, a single 115 kDa band (see [3]) at the same position as that for the isolated PLAP standard was identified as alkaline phosphatase by a colorimetric reaction [3] (data not shown). This confirmed earlier work using ^{3}H-fatty acid-labelled HEp-2 cells, where b-PLC released ^{3}H-labelled PLAP that could be immunoprecipitated with a specific monoclonal antibody and ran as a 66 kDa monomer on reducing SDS-PAGE gels [4].

3.2. *PLAP release following treatment with a GTP analogue*

Treatment of permealised HEp-2 cells with the non-hydrolysable analogue GTPγS produced a dose dependent increase in PLAP release (Fig. 1). A concentration of 100 μM GTPγS or greater was required to produce a significant increase in PLAP release. In comparison, treatment with GDPβS had little effect but did give values consistently higher than buffer alone that were about 20–25% of PLAP released following treatment with an optimal concentration of GTPγS. The actual amount of PLAP released calculated as mU/10^6 cells was variable between experiments (which appeared to be partly due to deterioration of the GTP analogue stock), but was always in the range of 0.8–5.0 mU/ml.

Table I

PLAP release following the treatment of HEp-2 cells with PLC

	PLAP released mU/10^{6}cells, ± SE
PBS alone	0.94 ± 0.27 (n = 6)
PI-PLC	49.0 ± 11 (n = 6)
PC-PLC	1.34 ± 0.37 (n = 5)

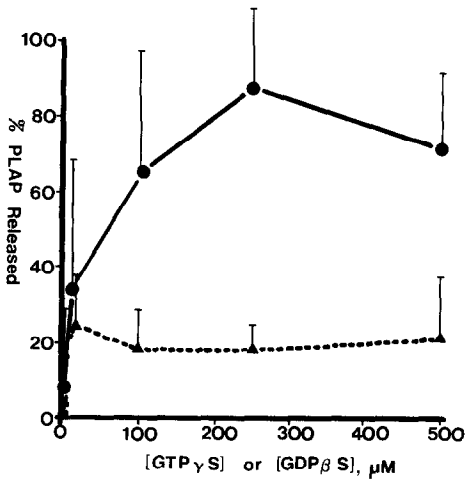

Fig. 1. The effect of GTPγS (●—●) and GDPβS (▲—▲) on PLAP release from digitonin-permealysed HEp-2 cells. The figure represents PLAP released as a % of the maximum GTPγS-stimulated PLAP release, the results being the mean of 5 experiments. Error bars are ± SE.

3.3. *PLAP release following treatment with growth factors*

The incubation of serum-starved HEp-2 cells with insulin or EGF produced a small but reproducibly measurable release of PLAP. PDGF in the range 1–100 ng/ml had no effect. The PLAP release following EGF or insulin treatment was dose-dependent and reached a plateau at 50 ng/ml EGF and 10 μg/ml insulin (Fig. 2a and b, respectively). The greatest release of PLAP was

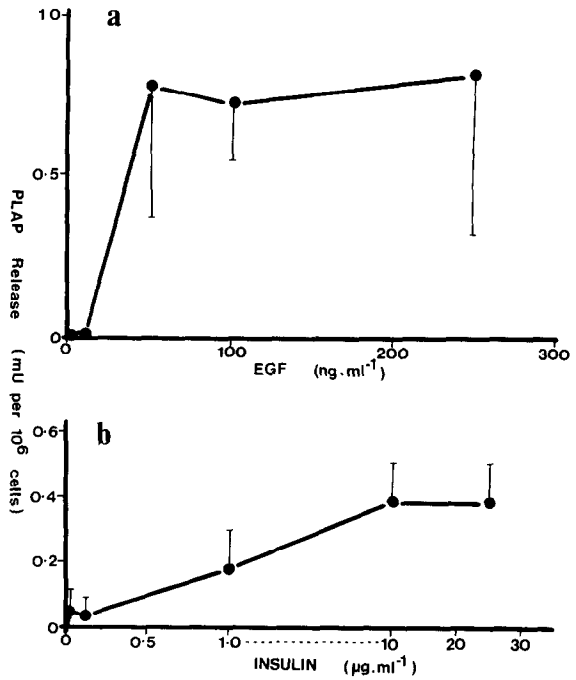

Fig. 2. Dose-response curves for (a) EGF- and (b) insulin-stimulated release of PLAP from intact HEp-2 cells. Error bars are ± SE, n = 5.

measured following treatment with EGF. In some experiments, half of the microfuged supernatant collected following treatment with EGF or insulin was filtered through 0.22 μMillipore filters to remove any remaining cell membrane contaminants. Both filtered and unfiltered samples gave the same values for PLAP release.

PLAP release from HEp-2 cells following growth factor treatment was less than that observed when cells were treated with GTPγS, the highest release with EGF being 0.82 ± 0.5 mU/10^6 cells and that with GTPγS 2.1 ± 1.6 mU/10^6 cells. This is in the range of 1.6–4.3% of surface PLAP releasable by exogenous b-PLC.

4. DISCUSSION

High levels of PLAP are found on the surface of human placental syncytiotrophoblast as well as certain tumour tissues and cell lines [1,3–5], while only trace amounts are found in normal, non-placental reproductive or other tissues [14]. PLAP is released into the maternal circulation as gestation progresses [1] and is also elevated in the serum of cigarette smokers and patients with PLAP-expressing tumours [1,16]. However, the physiological significance of PLAP expression and release remains obscure. In this study, we have investigated whether PLAP can be released from a tumour cell line by agents known to be capable of activating endogenous PLC as part of intracellular signalling mechanisms.

Treatment of permeabilised cells with non-hydrolysable analogues of GTP can permanently activate G proteins coupled to PLC, resulting in sustained hydrolysis of PI [9]. We have shown that a non-hydrolysable GTP analogue consistently released a small percentage of the total PLAP from permeabilised HEp-2 cells, indicating that activation of membrane-associated PLC by GTP could cleave this GPI-linked protein from the plasma membrane. The observation that GDPβS also induced a small increase in PLAP release over control values, although considerably less than that with GTPγS, does not appear consistent with the concept that GDP analogues inactivate G proteins and inhibit PI turnover. However, in this system GDPβS may inactivate a PLC-inhibitory G protein, releasing PLC from inhibitory control and resulting in raised PLC activity [16]. An increase of PLC activity with GDPβS was also recently reported in rat hepatocyte plasma membrane preparations [17].

Insulin, PDGF and EGF receptors have cytoplasmic tyrosyl kinase domains capable of stimulating PI turnover and activation of PLC in rapid response to ligand binding [18–20]. EGF and PDGF RTKs can directly phophorylate a PLC enzyme, PLC-II, suggesting the mechanism for their activation of this membrane-associated enzyme [11–13]. In this study, the incubation of HEp-2 cells with either insulin or EGF induced PLAP release from intact cells, indicating that PLC had been activated. This was not observed with PDGF treatment, but the expression of PDGF receptors by HEp-2 cells has not been confirmed. Insulin is unusual in that it promotes hydrolysis of a PI-glycan rather than PI 4,5-biphosphate by activation of a specific PLC to yield two products, a PI-glycan polar head group and diacylglycerol [21]. In this case, the growth factor RTK complex might induce tyrosine phosphorylation of G proteins, as has been demonstrated for insulin in vitro [22], although this has not been reported in vivo. Insulin has also been shown to induce the selective release of a number of other GPI-anchored proteins, including lipoprotein lipase [23], non-placental alkaline phosphatase [24] and two unidentified proteins of 35 kDa and 130 kDa [25] from insulin-sensitive cell lines, but the effect of EGF on release of these proteins has not been studied.

Only a small percentage of the total HEp-2 cell surface PLAP is released following incubation with EGF or insulin. Growth factor binding may activate only a small population of the total cellular PLC, or only a small proportion of activated PLC molecules may be able to release GPI-linked proteins. It would be of interest to assess the release of other GPI-linked cells surface proteins to determine whether any are more susceptible to release by growth factors than others, as suggested by Lisanti et al. [25]. Many forms of PLC have been identified in mammalian cells, of which there are at least 5 immunologically distinct forms and 3 have been sequenced and are known to be distinct gene products [13,26].

In conclusion, this study has demonstrated a role for G proteins, which are known activators of PLC, in PLAP release from tumour cells. Both insulin and EGF have been shown also to induce PLAP release from HEp-2 cells, presumably by the activation of PLC, although their mechanism of activation could be different.

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